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Published in:
Enzyme and Microbial Technology

DOI:
[10.1016/j.enzmictec.2003.08.007](https://doi.org/10.1016/j.enzmictec.2003.08.007)

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Document Version
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Publication date:
2005

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Citation for published version (APA):

Baldascini, H., & Janssen, D. B. (2005). Interfacial inactivation of epoxide hydrolase in a two-liquid-phase system. *Enzyme and Microbial Technology*, 36(2-3), 285 - 293.
<https://doi.org/10.1016/j.enzmictec.2003.08.007>

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Interfacial inactivation of epoxide hydrolase in a two-liquid-phase system

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Received 18 March 2003; received in revised form 27 August 2003; accepted 29 August 2003

Abstract

Enantioselective epoxide hydrolases are useful biocatalysts for the preparation of enantiopure epoxides and diols. The kinetic resolution of racemic epoxides can be carried out in an organic/aqueous biphasic system to allow use of high epoxide concentrations. Enzyme inactivation in such a system, however, may occur by contact with the interface. In this study, we investigated the factors which influence the interfacial inactivation of *Agrobacterium radiobacter* epoxide hydrolase in an octane/water biphasic system. Rates of interfacial inactivation were measured both in a stirred-cell, which has a planar interface, and in an emulsion reactor. Interfacial inactivation rates measured in the stirred-cell at a fixed interfacial area increased with mixing intensity. Interfacial inactivation rates per unit area were lower in the emulsion reactor than in the stirred-cell and increased with bulk aqueous enzyme concentration. Circular dichroism measurements showed that during biphasic incubation all unadsorbed soluble enzyme existed in the native conformation. Activity assays showed that the dissolved enzyme was also fully active, indicating that inactivated enzyme precipitated from solution. Using an inactive epoxide hydrolase mutant structurally similar to the wild-type enzyme in order to avoid the conversion of the epoxide, it was found that high concentrations of epoxide in the organic phase increased the rate of interfacial inactivation.

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Keywords: Enzyme stability; Interfacial inactivation; Liquid/liquid interface; Epoxide hydrolase; Styrene oxide

1. Introduction

Organic/aqueous biphasic mixtures can be used to increase the productivity of biocatalytic reactions when substrates are poorly soluble in water [1]. The kinetic resolution of racemic epoxides by the epoxide hydrolase from *Agrobacterium radiobacter* AD1 can be successfully carried out to produce aromatic (S)-epoxides of high enantiomeric excess in a buffer/octane emulsion system. High epoxide organic phase concentrations can be used, although enzyme stability is not sufficient to permit reuse in successive batches [2]. A preliminary analysis of enzyme stability in this system indi-

cated that inactivation was mainly caused by direct contact of the enzyme with the octane/water interface, rather than by the interaction of dissolved octane and enzyme on a molecular level [2]. Since large interfacial contact areas are required to ensure rapid mass transfer in the system, a detailed study of the factors that affect epoxide hydrolase interfacial inactivation under relevant operating conditions is required.

Enzyme susceptibility to interfacial inactivation is difficult to predict. Factors such as solvent polarity, interfacial tension [3], aqueous phase pH [4] and ionic strength [5,6] affect the extent of interfacial adsorption. The degree of enzyme structural rearrangement at an interface can be monitored with a variety of spectrophotometric techniques [7–9] and is dependent on the solvent properties mentioned above, as well as surface load. Furthermore, properties of the protein such as secondary structure [8], adiabatic compressibility [3], surface hydrophobicity, and thermostability [5,7] are also correlated with the sensitivity to interfacial inactivation.

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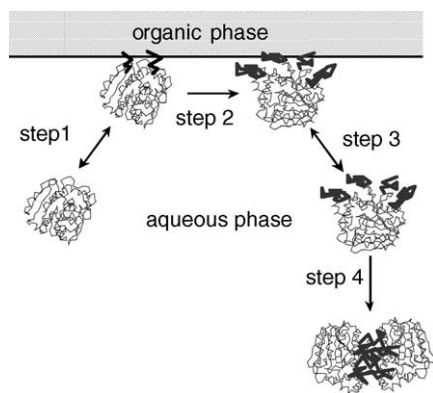


Fig. 1. Schematic representation of the proposed mechanism of enzyme inactivation at an aqueous/organic interface. Step 1: reversible enzyme adsorption to the interface and concomitant enzyme structural rearrangement at the interface; step 2: further relatively fast unfolding of enzyme molecule at the interface; step 3: rate-limiting desorption of inactivated/unfolded enzyme molecules from the interface; step 4: irreversible aggregation and precipitation of inactivated enzyme. Hydrophobic segments that become exposed during interaction with the interface and that are involved in aggregation are shown in bold as excursions from the main structure of the protein.

Interfacial inactivation can occur by enzyme adsorption to the interface followed by enzyme structural rearrangement (Fig. 1, steps 1 and 2) [10,11]. Studies on protein interfacial adsorption and inactivation are frequently carried out under quiescent conditions or in discontinuous systems in order to determine adsorption kinetics and adsorption isotherms. However, the complete dynamic equilibrium which is established, including enzyme desorption from the interface (Fig. 1, step 3), should be considered for describing overall rates of enzyme interfacial inactivation in real biphasic reactor systems. In a bubble column reactor, where the organic phase is bubbled through the aqueous phase, the enzyme solution is continuously exposed to a clean ('new') aqueous/organic interface to which no enzyme is initially adsorbed. For this type of contactor, it has been shown that the amount of enzyme inactivated is proportional to the total interfacial area of solvent introduced [12,13]. In emulsion reactors, however, the rate of creation of 'new' interface, and thus the observed rate of enzyme inactivation, is not so well controlled. It is determined by the frequencies of droplet break-up and coalescence, which are a function of interfacial tension, liquid density difference, dispersed phase volume fraction, mixing intensity and reactor geometry [14,15]. Furthermore, the creation of 'new' interfacial area will also be affected by the rate of enzyme desorption from the interface if active enzyme in solution replaces the desorbed enzyme [16], and this may be influenced by hydrodynamics.

In order to obtain more insight in the process of interfacial inactivation, we have measured rates of inactivation of the epoxide hydrolase from *A. radiobacter* in biphasic incubations under various conditions. The effect of mixing intensity on the rate of enzyme interfacial inactivation was investigated using a stirred-cell contactor. In this set-up, a fixed plate with a circular opening between the two liquids stabilises

and fixes the plane interface, allowing the mixing intensity in the aqueous phase to be varied independently of the interfacial contact area. Inactivation rates were also measured in an emulsion reactor under mixing conditions typically used for epoxide resolution experiments. Results showed that addition of epoxide to the organic phase increased enzyme interfacial inactivation.

2. Materials and methods

2.1. Chemicals and enzyme

Wild-type and mutant epoxide hydrolase preparations used in this work were produced as described by Rink et al. [17]. Purification was carried out with a DE52 anion exchange column. The partially purified enzyme was dialysed against TEMAG buffer (25 mM Tris- SO_4 , pH 7.5, 1 mM EDTA, 1 mM β -mercaptoethanol, 0.02% sodium azide and 10% glycerol), concentrated to 14 mg ml⁻¹ and stored at 4 °C. BSA powder (Fraction V, 96–99% albumin) was obtained from Sigma.

p-Nitrostyrene oxide (*p*NSO) was synthesised as described elsewhere [18]. *N*-octane (p.a. grade) was obtained from Fluka (Buchs, Switzerland) and was saturated with the aqueous phase buffer at 30 °C prior to use. The buffer composition in all experiments was 50 mM Tris- SO_4 , 1 mM EDTA, 1 mM β -mercaptoethanol, pH 7.5, unless otherwise stated.

2.2. Epoxide hydrolase activity assay

Enzyme activity assays were performed in a Perkin-Elmer Lambda Bio 40 UV/vis spectrophotometer with a temperature-controlled cell holder, by following the hydrolysis of the colorimetric substrate *p*NSO to its corresponding diol at 310 nm, for which extinction coefficients are $\epsilon_{310} = 4289 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{310} = 3304 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. Typically, 1 ml of enzyme solution at an appropriate concentration was placed in a 1 cm quartz cuvette and the reaction was started by adding (R)-*p*NSO or racemic *p*NSO (dissolved in acetonitrile). The concentration of acetonitrile was kept below 1% v/v since at high concentrations it is a competitive inhibitor of epoxide hydrolase. Conversion curves were either numerically fitted to the Michaelis–Menten equation to determine k_{cat} and K_{m} (for (R)-*p*NSO as substrate) using the software program Scientist (Micromath, Salt Lake City, UT) or used to obtain initial rates.

2.3. Enzyme stability in buffer

Stability of epoxide hydrolase was tested at 30 °C in 50 mM Tris buffer at pH 7.5 with additions of 1 mM β -mercaptoethanol and 1 mM EDTA, either separately or together. Stability was also measured with both additives at pH values of 7.5, 8, 8.5 and 9. For all incubations, enzyme

activity was assayed periodically using racemic *p*NSO. Inactivation was described by a first-order process. First-order inactivation rate constants, k_d , were fitted using a least squares minimisation procedure, from which enzyme half-life values were calculated using $t_{1/2} = \ln(2)/k_d$.

2.4. Stirred-cell contactor

A stirred-cell was used to measure rates of epoxide hydrolase inactivation in solutions contacted with octane while mixing at various stirring rates. The stirred-cell consisted of a thermostatted cylindrical vessel (7.5 cm diameter, 4.4 cm height) with two compartments separated by an interfacial plate. The bottom compartment was filled with 97 ml of enzyme buffer solution ($0.0084 \text{ mg ml}^{-1}$) and an equal volume of octane was contained in the upper compartment. The interfacial plate was attached to four baffles (0.7 cm width) positioned at the edge of the plate near the vessel walls, and had a central hole (5.2 cm diameter, 0.0022 m^2 area) to allow contact between the two phases. Both phases were stirred independently at equal rates (counter-current) by Rushton impellers (3.8 cm diameter, 0.7 cm blade width, 0.7 cm blade height) mounted in the middle of the compartments.

Aqueous phase mass transfer coefficients, k_a , were determined by following the transfer of a model substrate, styrene oxide, from the organic phase to the aqueous phase in the stirred-cell at 30°C , at stirring rates of 80, 150 and 190 rpm (stirrers rotating counter-currently at equal speeds in both phases). Values of the mass transfer coefficients and standard deviations were obtained by fitting the aqueous phase styrene oxide concentration profiles using a least squares minimisation procedure as described elsewhere [2].

2.5. Enzyme inactivation

2.5.1. Shear-induced inactivation

The effect of shear due to mixing on enzyme stability was measured by incubating an aqueous enzyme solution ($0.0093 \text{ mg ml}^{-1}$) in the stirred-cell reactor, kept at 30°C , which was stirred with one Rushton turbine at rates of 200 and 300 rpm. The reactor was completely filled with the enzyme buffer solution to eliminate any air/water interfaces. Enzyme activity was determined periodically by the colorimetric assay already described. The stability of enzyme incubated in buffer, left unstirred, was measured for comparison.

2.5.2. Interfacial inactivation

Rates of inactivation of epoxide hydrolase incubated in the stirred-cell were measured to determine the effect of mixing rate on inactivation rate. Experiments were carried out at an enzyme concentration of $0.0084 \text{ mg ml}^{-1}$, at stirring rates of 50, 150 or 180 rpm. In each experiment, stirring was increased carefully to the desired value to avoid breaking the flat interface. Periodically, 0.5 ml samples were taken from the aqueous phase, diluted by adding 0.7 ml of buffer, and remaining enzyme activity was determined by the colorimetric

assay already described. The liquid removed during sampling was replaced by buffer in order to keep the interface at the height of the interfacial plate. The decrease in enzyme activity with time, after correction for dilution due to sampling, was described by a first-order rate constant, k_d , and values were obtained by fitting the data using a least squares minimisation procedure. Decrease in activity of a control incubation of enzyme in buffer (unstirred) was measured for comparison.

Enzyme inactivation rates were also measured in an octane/buffer emulsion system. The reactor used was the same cylindrical vessel of the stirred-cell containing four baffles but without the interfacial plate. An emulsion was created by adding both liquid phases to the cylindrical vessel and stirring with one Rushton impeller (dimensions as above) placed at half the total liquid height. The emulsion had a total volume of 150 ml with an organic volume phase ratio of 0.2, and was stirred at 200 rpm. This reactor set-up and operating conditions have previously been used in styrene oxide kinetic resolution experiments using a biphasic system [2]. Inactivation rates were measured at initial enzyme concentrations of 0.0093 and 0.1 mg ml^{-1} . All interfacial inactivation experiments were conducted at 30°C .

2.6. Circular dichroism

Changes in epoxide hydrolase secondary structure were followed for a 0.1 mg ml^{-1} enzyme solution incubated in an emulsion system (at 30°C) as described above. Far-UV CD spectra were recorded on an AVIV circular dichroism spectrometer (62ADS) by measuring the change in ellipticity in millidegrees. Samples of the aqueous phase were taken directly from the emulsion system at various times and spectra were recorded at 25°C from 190–250 nm in a 1 mm cuvette. At later times during incubation, the samples were centrifuged at 13,000 rpm for 3 min to remove white precipitate-like particles prior to recording spectra. The spectra presented are the average of three scans using a bandwidth of 1 nm, a step width of 1 nm and 5-s averaging per point. The spectra were corrected for buffer signal. As a control, CD spectra were also recorded for an unstirred enzyme solution (0.1 mg ml^{-1}) in buffer with no octane present, kept at 30°C .

2.7. Effect of BSA addition on inactivation rate

The effect of addition of BSA to a buffer/octane emulsion on the rate of enzyme interfacial inactivation was tested. In 30 ml screw cap bottles, 14 ml of an epoxide hydrolase solution in buffer (0.013 mg ml^{-1}) were contacted with 6 ml of octane. The emulsions were kept at 30°C and mixed with a 2 cm long magnetic stirrer at approximately 300 rpm. BSA was added to the aqueous phase (0.1 mg ml^{-1}) either together with epoxide hydrolase before mixing was started, or soon (5 min) after emulsification of octane with an epoxide hydrolase solution was started. Enzyme activity was measured periodically by the colorimetric assay.

2.8. Enzyme interfacial inactivation due to epoxide in octane

The effect of adding styrene oxide to the octane phase on the rate of interfacial inactivation was studied by following precipitation of an inactive epoxide hydrolase mutant (Y152F + Y215F) from an aqueous buffer solution which was vigorously mixed with octane containing various concentrations of styrene oxide. The screw cap bottle emulsion set-up described above was used for these experiments. Solutions of 0.1 mg ml^{-1} mutant epoxide hydrolase were contacted with octane containing either 0, 25, 250 or 600 mM styrene oxide. The emulsions were kept at 30°C and mixed with a 2 cm long magnetic stirrer at 300 rpm. For comparison, the mutant enzyme was also incubated in buffer containing 0 or 6 mM (dissolved) styrene oxide (no octane) and stirred as the emulsion incubations. Periodically, soluble enzyme concentration was measured by the Bradford assay. Samples of $100 \mu\text{l}$ were taken from the aqueous phase and filtered by centrifugal filtration using ultrafree-MC microporous devices with a $0.2 \mu\text{m}$ pore size (Millipore BV), prior to addition to 1 ml Bradford reagent and measurement of adsorption at 595 nm.

3. Results and discussion

3.1. Enzyme stability in buffer

In order to investigate enzyme interfacial inactivation independently of other inactivation processes, we minimised enzyme inactivation in the aqueous phase by appropriate choice of pH and by adding mercaptoethanol and EDTA, compounds that are commonly used to stabilise enzymes [19]. In 50 mM Tris buffer at pH 7.5 and 30°C with no additives, the epoxide hydrolase had a half-life of 5.5 days. The half-life was increased to approximately 20 days by the addition of 1 mM EDTA and to 22 days by further addition of 1 mM mercaptoethanol. Optimum pH for enzyme stability was determined in buffer containing both the additives at pH values between 7.5 and 9. Enzyme stability was highest at pH 7.5; with a half-life approximately double that at pH 9. The selected aqueous phase composi-

tion for further experiments was 50 mM Tris, pH 7.5, 1 mM β -mercaptoethanol and 1 mM EDTA, where the enzyme showed remarkable stability with a half-life of approximately 24 days ($k_d = 1.95 \times 10^{-5} \pm 8.3 \times 10^{-6} \text{ min}^{-1}$).

The effect of the shear forces created by mixing with the Rushton turbine on enzyme stability, in the absence of a liquid/liquid or air/liquid interface, was determined. Comparison with an unstirred enzyme solution showed that no significant shear-induced inactivation occurred over 150 h (data not shown). We conclude that in this reactor set-up shear-induced inactivation at an enzyme concentration of $0.0093 \text{ mg ml}^{-1}$ and below a stirring rate of 300 rpm is neglectable.

3.2. Interfacial inactivation in the stirred-cell contactor

A stirred-cell was used to determine whether shear forces resulting from mixing acting at the interface influence the overall interfacial inactivation rate. This was done by measuring enzyme inactivation rates at stirring rates of 50, 150 and 180 rpm. Stirring rates were restricted to 50–190 rpm since above 190 rpm excessive rippling disturbed the interface. Below 190 rpm, the increase in interfacial area due to surface rippling was estimated to be less than 5%.

At the different stirring speeds, remaining enzyme activity was followed for 150 h. The enzyme activity decreased in time. The rate of enzyme inactivation decreased in time and could be described with a first-order inactivation rate constant, k_d . Enzyme inactivation rates increased with increasing stirring rate (Table 1, Fig. 2). Although epoxide hydrolase inactivation by a molecular mechanism, where dissolved octane interferes with enzyme integrity, is minor compared to that which occurs by the interfacial mechanism [2], its contribution should be taken into account when comparing inactivation rates measured at different mixing rates. Assuming that the contributions of the molecular and interfacial mechanisms to the overall inactivation are additive, the first-order rate constant for interfacial inactivation (k_i) was calculated by subtracting the first-order inactivation rate constant for molecular inactivation ($1.6 \times 10^{-5} \text{ min}^{-1}$ [2]) from the observed inactivation rate constant (k_d) in each experiment. Initial rates of interfacial inactivation per unit area of interface present in the system (initial specific interfacial inactivation rate)

Table 1
Effect of different mixing conditions on interfacial inactivation of epoxide hydrolase

Incubation (reactor type)	Stirring rate (rpm)	Enzyme concentration (mg ml^{-1})	Aqueous phase volume (ml)	Total interfacial area (m^2)	$k_d (\times 10^{-5} \text{ min}^{-1})$
Stirred-cell	50	0.0084	97	0.0022	3.4 ± 0.46
Stirred-cell	150	0.0084	97	0.0022	4.2 ± 0.39
Stirred-cell	180	0.0084	97	0.0022	6.4 ± 0.56
Emulsion	200	0.0093	120	0.045	9.9 ± 0.84
Emulsion	200	0.10	120	0.045	4.1 ± 0.36
Control	0	0.0084	—	0	1.0 ± 0.70
Control	0	0.10	—	0	0.56 ± 0.18

Enzyme was incubated in a stirred-cell, using various stirring speeds, or in an emulsion reactor at different enzyme concentrations. Enzyme incubated in an unstirred buffer solution was used as a control. Inactivation was measured by following the remaining enzyme activity in time. k_d is the measured first-order inactivation rate constant.

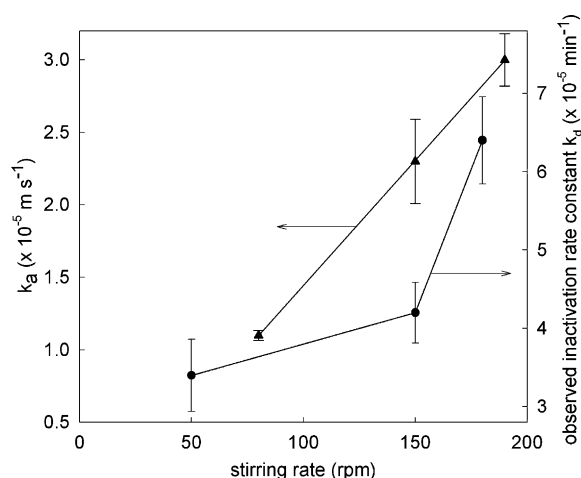


Fig. 2. Interfacial epoxide hydrolase inactivation by contact with an octane/water interface in the stirred-cell. Effect of stirring rate on (▲) the aqueous phase mass transfer coefficient k_a for transfer of styrene oxide from the organic to the aqueous phase, and on (●) the observed first-order rate constant for epoxide hydrolase inactivation. Error bars show standard deviations obtained for fitting a single dataset. The initial enzyme concentration was fixed at 0.0084 mg/ml and the interfacial area was fixed at 0.0022 m².

were calculated to allow comparison between the different biphasic incubations. These were calculated by the formula, $k_i[E]_0V_{aq}/A_{int}$, where $[E]_0$ is the initial enzyme concentration in the aqueous phase, V_{aq} the aqueous phase volume and A_{int} is the total interfacial area in the system. The initial specific interfacial inactivation rates were 6.6×10^{-3} , 9.6×10^{-3} and $1.8 \times 10^{-2} \text{ mg min}^{-1} \text{ m}^{-2}$ for the stirring rates of 50, 150 and 180 rpm, respectively.

To account for the extent of inactivation observed in the experiments, an exchange between inactive enzyme molecules at the interface and active enzyme molecules from solution must take place. This is because, assuming that an epoxide hydrolase molecule is a sphere of 48 Å diameter [20], a closely packed monolayer coverage of the interface would require a surface load of 3.1 mg m^{-2} . Thus, monolayer coverage requires only a small fraction of the total enzyme present. The increase in the observed inactivation rates with mixing intensity should be explained by considering the relative rates of enzyme adsorption, inactivation and desorption in the interfacial inactivation process.

During the inactivation process, enzyme is first transported to the interface subsurface by convection and diffusion. At the interface, enzyme molecules adsorb and undergo a small structural perturbation (Fig. 1, step 1), possibly accompanied by loss of activity. This perturbation, and thus also adsorption, is reversible [21]. The rate of enzyme adsorption is proportional to the aqueous enzyme concentration at the interface subsurface [22]. Adsorption rates of $36 \text{ mg min}^{-1} \text{ m}^{-2}$ have been found for proteins adsorbing to an air/liquid interface from an enzyme solution of 0.1 mg ml^{-1} [12].

Adsorbed enzyme molecules undergo further irreversible structural rearrangement (Fig. 1, step 2) due to attraction between the hydrophobic enzyme core and the hydrophobic

interface [10]. Complete unfolding of the enzyme molecules may require long contact times [8,23], implying that a range of unfolded enzyme conformations can be present at the interface at any time. Lateral interactions between partially unfolded enzyme molecules adsorbed at interfaces may occur [24] and depend on surface coverage and extent of unfolding of the enzyme molecules. The extent of unfolding itself has been shown to decrease with increasing surface coverage [8,11,25].

In step 3, unfolded/inactive enzyme desorbs from the interface. Whereas the adsorption step is recognised as a spontaneous process [10], desorption of unfolded enzyme molecules from the interface does not occur spontaneously, for example by simple dilution of the aqueous phase, since it requires the disruption of the entropically favourable interactions between the enzyme and the solvent at the interface [11,21,22]. Estimation of the rate of enzyme transport to the interface subsurface shows that it is not rate-limiting, therefore, we suggest that the desorption of inactivated enzyme from the interface is the rate-limiting step in the inactivation process occurring in the stirred-cell contactor. At steady state, the interface is completely covered by native and unfolded enzyme, and the net rate of adsorption of native enzyme molecules from solution is equal to the rate of desorption of inactivated molecules. The increase in specific inactivation rate with stirring speed observed in these experiments would then suggest that increasing the mixing intensity increases the rate of desorption. The rippling effects observed at the interface were ascribed to motion of the interface perpendicular to the plane of the interface rather than expansion and contraction of the interface along the interfacial plane. The effect of this interface motion would be to increase the total interfacial area in the stirred-cell, which was estimated as less than 5% at 180 rpm, rather than creation of 'new interfacial' area in time. Therefore, the increased rate of interfacial inactivation cannot be explained by greater rippling motion at the interface. We conclude that the desorption of inactivated enzyme molecules from the interface is caused by impingement of circulating eddies at the interface, whereby an increase in mixing intensity increases the rate of surface renewal and the magnitude of forces acting at the interface.

The aqueous phase mass transfer coefficient, k_a , for transfer of styrene oxide between the two phases was determined at the different stirring rates as a direct measure of mixing intensity in the aqueous phase. The k_a values increased linearly with increasing stirring rate (Fig. 2). The specific interfacial inactivation rates, however, increased more than proportionally with stirring speed. This different dependency on the stirring rate indicates that the rate of transfer of enzyme molecules to the interface subsurface is indeed not rate-limiting, otherwise a linear increase in specific inactivation rate with stirring rate would also be expected. According to surface renewal theory of mass transfer, surface renewal rates at interfaces vary exponentially with mixing rates when the interface has a layer of adsorbed material because eddies require a certain momentum to clear an area at the interface

[26]. This suggests that the effectiveness of eddy-assisted enzyme desorption increases as the turbulence of mixing increases.

3.3. Interfacial inactivation in an octane/water emulsion

Successful enzymatic kinetic resolutions of epoxides in a biphasic system have been carried out in emulsions since high interfacial areas are required to ensure rapid interphase substrate mass transfer [2]. Therefore, we also measured interfacial inactivation rates in an emulsion system typically used for these reactions. The total interfacial area in the emulsion set-up was calculated as 0.045 m^2 by visually estimating organic phase droplet sizes (0.4 cm diameter), corresponding to a 16-fold larger specific interfacial area compared to the stirred-cell set-up. The contribution of molecular inactivation due to dissolved octane to the total inactivation measured was taken into account as for the stirred-cell experiments. The initial specific interfacial inactivation rate for an enzyme aqueous phase concentration of $0.0093 \text{ mg ml}^{-1}$ was calculated as $2.0 \times 10^{-3} \text{ mg min}^{-1} \text{ m}^{-2}$. This value is approximately 10 times lower than the highest specific inactivation rate found in the stirred-cell experiment operated at 180 rpm.

Comparison of the specific interfacial inactivation rates in the two systems shows that the rate of creation of 'new' interface to which enzyme from solution can adsorb is lower in the emulsion system than in the stirred-cell. In the emulsion system at steady state, 'new' interface is created by two mechanisms: by desorption of inactivated enzyme molecules from the interface, as also occurs in the stirred-cell, and additionally by the dynamic process of droplet coalescence and break-up since the surface which is created upon droplet break-up is initially free of adsorbed enzyme. The contribution of this latter mechanism to the creation of new interface in the emulsion may in fact be low since enzyme adsorbed at the interface can hinder droplet coalescence [15]. Furthermore, the rate of desorption of inactivated enzyme molecules from the interface may also be lower in the emulsion compared to the stirred-cell since droplets freely follow flow patterns in the aqueous phase, possibly resulting in lower shear stresses at the interface. We conclude that emulsions with high interfacial areas can be exploited to obtain high solute interphase mass transfer rates with relatively low enzyme inactivation, since the interfacial inactivation rate is mostly determined by the rate of exchange between inactive and active enzyme molecules at the interface, rather than by the absolute amount of interface present in the system.

The effect of enzyme concentration on inactivation rate was investigated at two different values in the emulsion system (Table 1), at a fixed mixing rate. At an aqueous enzyme concentration of 0.1 mg ml^{-1} , the initial specific interfacial inactivation rate was calculated as $6.7 \times 10^{-3} \text{ mg min}^{-1} \text{ m}^{-2}$, which is approximately three-fold higher than what was found at $0.0093 \text{ mg ml}^{-1}$. This value was calculated assuming that the first-order rate constant for inactivation due to molecular toxicity of octane does not change with the

bulk aqueous enzyme concentration, and is based on a total interfacial area of 0.045 m^2 . The increase in specific interfacial inactivation rate with enzyme concentration suggests that the enzyme surface load at full interfacial coverage is higher when the bulk aqueous enzyme concentration is higher. This would be possible if the rate of adsorption (Fig. 1, step 1) increases relative to the rate of unfolding (Fig. 1, step 2), so that adsorbed and inactivated enzyme molecules exist in a more compact unfolded state at the interface. At the higher surface load, a larger number of inactive enzyme molecules would be desorbed per eddy clearance at the interface even though the rate of surface renewal remains constant since mixing rate is unchanged.

3.4. Epoxide hydrolase structural changes during interfacial inactivation

To further examine the loss of enzyme activity by interfacial contact, changes in enzyme structure during biphasic incubation were monitored by recording far-UV circular dichroism spectra of aqueous phase samples taken from an emulsion system at different points in time. Enzyme was present at an initial aqueous phase concentration of 0.1 mg ml^{-1} . In parallel, remaining enzyme activity was measured by the colorimetric assay.

Enzyme inactivation was accompanied by the formation of a white particulate precipitate in the aqueous phase. The decrease in soluble enzyme concentration in time, determined by the change in CD signal intensity at 220 nm, measured after sample centrifugation, indicated that the white particles were precipitated enzyme aggregates. Comparison of the shape of the far-UV CD spectra recorded from the emulsion incubation and from a control incubation (no octane), after correction of signal intensity for the decrease in soluble enzyme concentration, showed that no change in the secondary structure of soluble enzyme took place over the whole period of incubation (Fig. 3). Therefore, all soluble unadsorbed enzymes had a native secondary structure.

Analysis of the kinetic data derived from the colorimetric activity assays showed that while the maximum rate of conversion of the substrate (R)-*p*NSO (U/ml of incubation mixture) decreased during the course of the incubation, the K_m of conversion remained the same. This also confirmed that no enzyme structural changes affecting the affinity of soluble enzyme for the substrate occurred during inactivation experiments. Furthermore, the decrease in soluble enzyme concentration correlated well with the measured decrease in enzyme activity (Fig. 4), from which it can be further concluded that all soluble enzyme remained catalytically active during biphasic incubation.

Since it is unlikely that unfolded enzyme molecules regain the native secondary structure after desorption from the hydrophobic liquid interface [9,11], aggregation of inactivated enzyme molecules (by interaction of their hydrophobic cores) with resultant precipitation from solution must occur before desorption or rapidly after desorption from the interface [11]

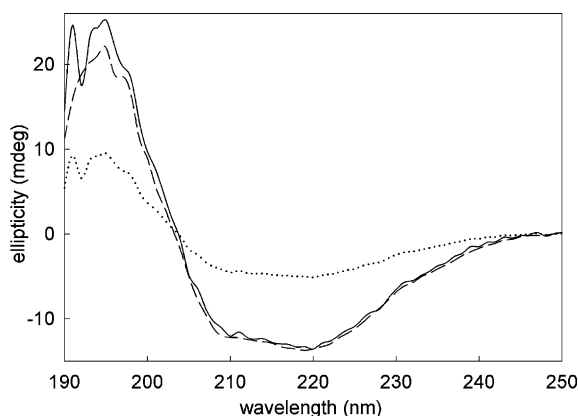


Fig. 3. Effect of interfacial inactivation in an octane/buffer emulsion on the far-UV CD spectrum of epoxide hydrolase. The CD spectrum of enzyme incubated in an octane/buffer emulsion, which was taken when enzyme activity had decreased by 65% after 359 h of incubation (dotted line), was compared to the spectrum of enzyme incubated in only buffer for the same time (control) (dashed line). The CD spectrum of the sample taken from the emulsion system adjusted for the decrease in enzyme concentration is also shown (full line).

(Fig. 1, step 4) because no unfolded soluble enzyme was detected in the aqueous phase.

3.5. Decreasing the rate of interfacial inactivation

Surfactants and macromolecular compounds have been widely tested for their ability to reduce protein interfacial inactivation. BSA is a protein frequently used for this purpose since it is highly surface-active [23]. Inactivation rates of epoxide hydrolase in a set of emulsion incubations were compared to determine whether addition of BSA could reduce the rate of epoxide hydrolase interfacial inactivation. The addition of BSA to the aqueous phase at a concentration of 0.1 mg ml^{-1} reduced the rate of interfacial inactivation of epoxide hydrolase considerably (Fig. 5). The order in which

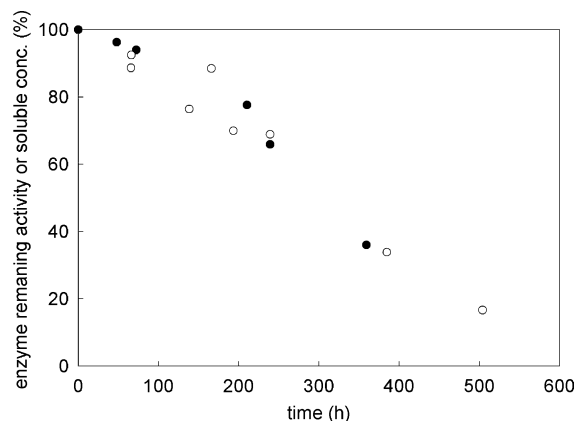


Fig. 4. Inactivation of epoxide hydrolase in the emulsion system. Comparison of remaining enzyme activity (○) (relative to a control incubation with no octane) determined by the colorimetric assay; and (●) the remaining enzyme concentration in the aqueous phase (relative to a control incubation with no octane) determined by decrease in CD signal intensity at 220 nm.

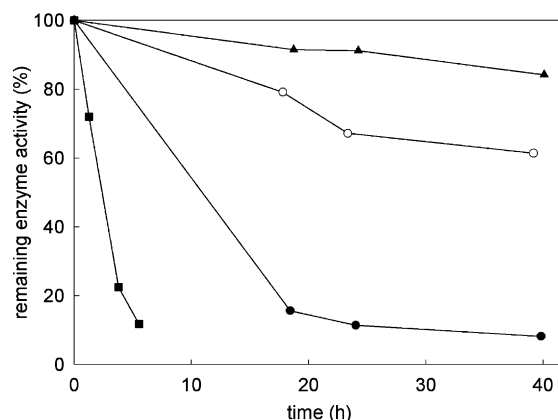


Fig. 5. Effect of BSA addition on the rate of epoxide hydrolase inactivation incubated in octane/buffer emulsions. The remaining enzyme activity in time is given for: (▲) a control enzyme incubation (no octane); (■) an emulsion incubation with no BSA present; (●) an emulsion incubation in which BSA was added to the system 0.08 h after emulsification with epoxide hydrolase had been initiated; and (○) an emulsion incubation in which BSA and epoxide hydrolase were added together before emulsification was initiated. All incubations had a total volume of 20 ml and were stirred at 300 rpm with a 2 cm magnetic stirrer. Emulsions had an organic phase ratio of 0.3 v/v. The aqueous phase was composed of 50 mM Tris, 1 mM EDTA and 1 mM β -mercaptoethanol containing BSA and epoxide hydrolase at 0.1 and 0.013 mg/ml, respectively.

the two proteins were added and emulsification was initiated greatly influenced the degree of interfacial protection provided. When BSA was added after emulsification had taken place, its protective effect was much less than when BSA was added before emulsification was initiated. This indicates that the observed protecting effect was not due to a general stabilising property of BSA but suggests that the observed reduction in inactivation rate of epoxide hydrolase occurred as a result of the competition in adsorption of the two proteins at the interface.

3.6. Effect of epoxide on interfacial inactivation

The experiments described above show that the rate of interfacial inactivation of epoxide hydrolase in an emulsion of pure octane and buffer is relatively low, and that at relevant enzyme concentrations enzyme half-life remains much higher than the reaction time of a typical batch biphasic kinetic resolution (10 h). However, the presence of epoxide substrate such as styrene oxide in octane at high concentrations, which is desirable for high process productivity, may cause an increase in the rate of enzyme inactivation [2]. Using the wild-type epoxide hydrolase, the effect of epoxides on enzyme interfacial inactivation cannot be measured independently from stability effects arising from the formation of diol. Preliminary tests have shown that incubation of epoxide hydrolase in buffer containing 100 mM 1-phenyl-1,2-ethanediol, the hydrolysis product of styrene oxide, reduces enzyme half-life from 24 days to approximately 24 h. Therefore, to study the effect of the presence of styrene oxide in the organic phase on epoxide hydrolase interfacial inactivation we used an inactive

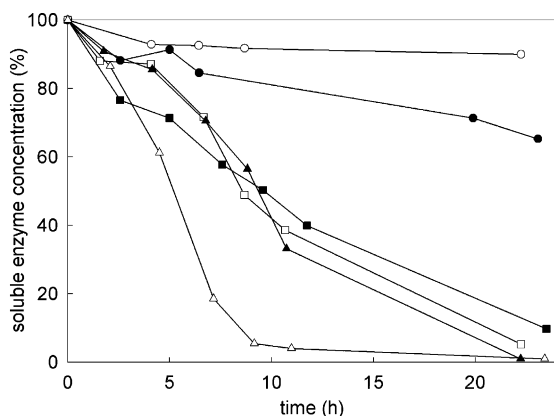


Fig. 6. Effect of styrene oxide in the organic phase on the rate of epoxide hydrolase inactivation. 14 ml of aqueous solutions of an inactive epoxide hydrolase mutant (Y215F + Y152F) at 0.1 mg/ml were contacted with 6 ml octane containing styrene oxide at varying concentrations. Change in soluble enzyme concentration was measured at each time point by the Bradford assay. Styrene oxide concentrations: (Δ) 600 mM in octane; (\blacktriangle) 250 mM in octane; (\square) 25 mM in octane; (\blacksquare) 0 mM in octane; (\bullet) 6 mM aqueous phase (no octane); and (\circ) 0 mM (no octane).

mutant of the epoxide hydrolase [27] in order to avoid the formation of diol during the experiment. The mutation of two tyrosine residues, normally situated in the active site, to phenylalanine (Y215F + Y152F) resulted in a six orders of magnitude decrease in k_{cat}/K_m values for conversion of styrene oxide, making the rate of diol formation by enzymatic conversion of the same magnitude as that by epoxide chemical hydrolysis. The concentration of diol formed during any incubation would be less than 7 mM, and tests have shown that this would cause negligible enzyme inactivation (data not shown). By CD spectroscopy, this mutant has been shown to have an identical overall structure to the wild-type enzyme [27], so we assume that it would undergo inactivation/unfolding upon adsorption by the same mechanism as wild-type enzyme. Using this mutant enzyme, interfacial inactivation rates in emulsions containing styrene oxide were measured by monitoring the decrease in dissolved enzyme concentration in time. This is justified since we have shown above that interfacial inactivation results in enzyme precipitation from solution.

The mutant enzyme was incubated in emulsions containing different styrene oxide concentrations in the octane phase. The emulsions were stirred with a magnetic stirrer giving organic phase droplets of approximately 0.4 μm diameter. Control incubations consisted of enzyme in aqueous buffer, with 0 or 6 mM dissolved styrene oxide, which were similarly stirred.

In all incubations, the soluble enzyme concentration decreased in time (Fig. 6). In the control incubation with no epoxide and no octane, stirring alone caused approximately 10% enzyme precipitation over the 24 h incubation period. This may have been largely due to enzyme denaturation at the air/water interface [16]. Precipitation of epoxide hydrolase in an aqueous solution of 6 mM styrene oxide was tested to ac-

count for enzyme inactivation due to molecular toxicity of styrene oxide. In this incubation, 30% enzyme precipitation occurred over the period of incubation (Fig. 6), which suggests that some precipitation can be caused by the molecular toxicity of styrene oxide. Nevertheless, enzyme inactivation in all biphasic incubations was faster than in the single aqueous phase incubation containing styrene oxide, indicating that inactivation in the biphasic systems was caused mainly by interfacial effects.

The presence of styrene oxide above a threshold concentration increased the rate of interfacial inactivation. At a concentration of styrene oxide in the octane phase of 600 mM, the inactivation was considerably faster than at 250 mM. However, below 250 mM the rate of interfacial inactivation did not appear to change with styrene oxide concentration. The increase in interfacial inactivation rate could be due to a decrease in interfacial tension, which is expected upon addition of styrene oxide. A lower interfacial tension would result in a lower average droplet diameter, and thus a greater steady-state interfacial area. This could not be confirmed experimentally since the droplet sizes could only be determined approximately. Why the effect is observed only above a threshold epoxide concentration remains unclear. A decrease in interfacial tension would in principle also increase the frequency of droplet coalescence and dispersion, resulting in a higher rate of creation of 'new' interface and thus a higher rate of interfacial inactivation.

4. Conclusions

Inactivation of epoxide hydrolase at the octane/water interface takes place by unfolding of enzyme molecules adsorbed at the interface, followed by enzyme aggregation and finally precipitation from solution. Increasing the mixing intensity was found to increase the rate of interfacial inactivation and we propose that this effect is due to an increase in the rate of desorption of inactivated enzyme molecules from the interface which then allows active enzyme in solution to become adsorbed and inactivate in turn.

By comparing interfacial inactivation rates in a stirred-cell and an emulsion system we have shown that the use of an emulsion system can be exploited to obtain high solute interphase mass transfer rates since the rate of specific interfacial inactivation remains low. However, in this system, the presence of epoxide substrate at high concentration in the organic phase increases the rate of interfacial inactivation. Addition of a sacrificial protein to the system, which can prevent adsorption of the catalytic enzyme at the interface, could provide a method to reduce the rate of interfacial inactivation.

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